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COMMENTARY

ANTIOXIDANT RESPONSE ELEMENT

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Antioxidant response element (ARE†), also referred to as electrophile response element (EpRE), has been found in the promoter regions of rat and human NAD(P)H:quinone oxidoreductase$_1$ (NQO_1/QR/QAO/DT diaphorase) genes and rat and mouse glutathione *S*-transferase Ya subunit (GST-Ya) genes; it has been shown to mediate high basal expression of these genes in tumor tissues, as compared with normal tissues of the same origin, and induction of expression in response to bifunctional [e.g. β-naphthoflavone (β-NF) and 3-methylcholanthrene (3-MC)] and monofunctional [e.g. *tert*-butylhydroquinone (tBH) and 2(3)-*tert*-butyl-4-hydroxyanisole (BHA)] inducers [1–5]. The mouse element has been named EpRE. An element designated GPEI, with characteristics similar to those of ARE, has also been identified in the promoter of the rat glutathione *S*-transferase P (GST-P) gene [6, 7]. In addition to the four inducers listed above, a variety of other chemicals, including Michael reaction acceptors, diphenols and quinones, isothiocyanates, peroxides, mercaptans, trivalent arsenicals, and heavy metal salts, have been shown to mediate induction through the mouse GST-Ya subunit gene EpRE (ARE) [8]. Interestingly, many of these compounds are substrates for GSTs and NQO_1 (DT diaphorase). The increase in the activities of NQO_1, GSTs and other phase II enzymes is known to provide protection against neoplastic, mutagenic, and other toxic effects of many carcinogens [9]. This protection is due to detoxification functions of phase II enzymes that include GSTs, which conjugate hydrophobic electrophiles with glutathione (GSH) [10–12]; UDP-glucuronosyl transferases (UDP-GT) [13], which catalyse the conjugation of glucuronic acid with xenobiotics and drugs for their excretion; epoxide hydrolase (EH) [14], which inactivates epoxides; and NAD(P)H:quinone oxidoreductases (NQOs), which catalyse obligatory two-electron reduction of quinones and their derivatives and prevent their participation in redox cycling and oxidative stress [15, 16]. The ARE sequences are also expected to be present in the promoters of EH and UDP-GT genes and are the subject of great interest. These observations strongly suggest the significance of ARE in chemoprevention, which has attracted much attention recently, primarily because of an increase in the incidence of chemical carcinogenesis due to the almost unavoidable exposure to several potential carcinogenic chemicals present in food, water and the environment. Enhanced detoxification and consequent elimination from the body by way of modulating the activities of phase II enzymes mediated through ARE are by far the best approach for chemoprevention [15, 17, 18]. At present, there are more questions than answers in regard to the sequence needed for optimal activity of ARE, different kinds of AREs, proteins binding to the ARE, and the mechanism of signal transduction through ARE. In this commentary, we have summarized the published results on the ARE and addressed several questions and controversies. Note that we have also used the term "ARE" for EpRE and GPEI, to simplify the discussion.

Presence of AP1 and AP1-like elements in ARE

The nucleotide sequences of AREs characterized in rat and human NQO_1 genes, rat GST-P gene, and rat and mouse GST-Ya genes are shown in Fig. 1. In some cases, the ARE sequences shown in Fig. 1 have been extended (in italics) to include more of the 5′- and 3′-nucleotides from the corresponding genes to better align them for analysis. These AREs contain two or more copies of the AP1 or AP1-like elements in a short stretch (40–45 nucleotides) of the DNA [1–8]. The AP1 element (TGACTCA), also known as TRE (TPA response element), present in the upstream regions of several genes including human metallothionein, SV40 and human collagenase genes, is known to increase the transcription of these genes in response to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [19, 20]. The AP1-like elements are imperfect AP1 binding consensus sequences. The human NQO_1 gene ARE contains one copy of the perfect consensus sequence for AP1

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† Abbreviations: ARE, antioxidant response element; XRE, xenobiotic response element; TRE, TPA response element; EpRE, electrophile response element; β-NF, β-naphthoflavone; BHA; 2(3)-*tert*-butyl-4-hydroxyanisole; tBH, *tert*-butylhydroquinone; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; NQO_1, NAD(P)H:quinone oxidoreductase$_1$, also known as quinone reductase (QR), quinone:(acceptor) oxidoreductase (QAO) and DT diaphorase (EC 1.6.99.2); GSTs, glutathione *S*-transferase.

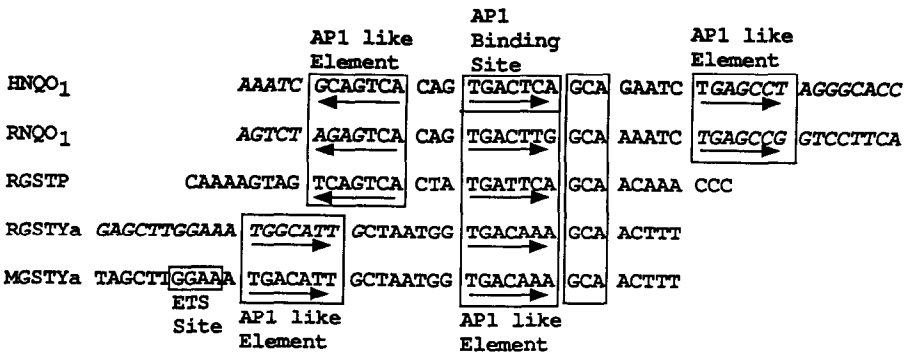

Fig. 1. Alignment of antioxidant response elements (AREs) from five genes: $HNQO_1$, human NQO_1 gene; $RNQO_1$, rat NQO_1/QR gene; RGSTP, rat GST-P gene; RGSTYa, rat GST-Ya subunit gene; and MGSTYa, mouse GST-Ya subunit gene. The sequences in italics are additional sequences from respective genes for alignment purposes. The AP1 and AP1-like elements and their orientations are indicated. The middle AP1 site in the human NQO_1 gene ARE is a perfect consensus sequence and has been separated from AP1-like (imperfect consensus) elements in all other genes. The 'GCA' box and ETS binding sites are also shown in boxes.

binding followed by the nucleotides 'GCA' ([21], Fig. 1). This is flanked by AP1-like elements on each side, making a total of three AP1/AP1-like elements in the human NQO_1 gene ARE. The first two AP1 elements are arranged in inverse orientation separated by three nucleotides and have been shown to respond to β-NF induction of a reporter gene in a manner similar to that seen with 1.55 kb upstream of the NQO_1 gene promoter [21]. The role of the 3′ AP1-like element remains unknown. It may be possible that all of the three AP1 elements are required for optimal expression and/or induction; this remains to be investigated. All these elements, their orientation and spacing are conserved between human and rat NQO_1 gene AREs, except that in the rat NQO_1 gene ARE, all three AP1 sites are imperfect AP1 (AP1-like) elements (Fig. 1). The rat GST-P gene ARE contains two AP1-like elements arranged in a manner similar to the 5′ and middle AP1-like elements in the human and rat NQO_1 genes ([22], Fig. 1). The rat and mouse GST-Ya subunit gene AREs contain two copies of the AP1-like elements arranged as direct repeats at an interval of 8 bp [8, 23]. The minimum ARE sequence required for expression and induction of rat GST-Ya and rat NQO_1 genes has been determined as 5′puGTGACNNNGC3′, which contains only one AP1-like element [24]. Fine mutational analysis in the rat GST-Ya gene ARE showed that 6 bp "TGAC***GC" are essential for induction, and 4 out of these 6 bp (TGAC) are also required for basal expression [24]. This is in contrast to the reports on rat GST-P gene and mouse GST-Ya subunit gene AREs, which require the presence of both of the AP1-like elements for their proper function [8, 23]. The GST-P gene ARE, containing two AP1-like elements, exhibits a strong transcriptional enhancing activity in F9 embryonal carcinoma and HeLa cells [22]. The mutation of one AP1-like element severely affects the activity of the ARE. In addition, Li and Jaiswal [21] observed that mutation of a perfect AP1 binding site in the human NQO_1 gene ARE results in the loss of basal and β-NF-induced expression. In summary, the AREs in various genes usually contain two AP1-like elements arranged in varying orientations, separated by either three or eight nucleotides. The orientations and spacing between the two AP1 elements could be important in determining the levels of basal and induced expression and are of great future interest. It will be interesting to determine if these differences could lead to significant changes in the mechanisms of induction mediated by various AREs. Questions also remain as to whether β-NF and BHA induce a classical TRE-controlled CAT reporter gene. Hydrogen peroxide (H_2O_2) has been shown to induce gene expression mediated by a perfect AP1 element (TRE) [25] and ARE [24–26] and may be an important intermediate in the induction of genes containing these elements. It is not clear if H_2O_2 induction of gene expression mediated by AP1 and ARE is controlled by similar or different mechanisms; this point remains to be investigated.

Additional DNA elements contained within the ARE have been shown to contribute to optimal expression and induction of GST-Ya subunit genes [8, 24]. Two nucleotides, 'GC', immediately following the 3′ AP1-like element within the rat GST-Ya gene ARE (Fig. 1), are not required for basal expression but are essential for induction [24]. These two nucleotides are part of the three nucleotides 'GCA', which are highly conserved in all kinds of AREs (Fig. 1). However, the roles of these nucleotides in NQO_1 and GST-P gene AREs are not known and should be investigated. In addition, Prestera *et al.* [8] reported the presence of an ETS protein binding site just before the 5′ AP1-like element. The mouse GST-Ya subunit gene ARE containing the two AP1-like elements but lacking the preceding ETS protein binding sequence was considerably less responsive to the same inducers.

The ETS binding sites have not been found in NQO_1 and GST-P gene AREs.

Interaction of ARE-regulatory proteins

It is an established fact that AP1 (c-Jun) and other related proteins bind to the AP1 binding site or TRE, present in the upstream regions of several genes (e.g. human metallothionein, SV40 and human collagenase) and increase the transcription of the respective genes in response to TPA [27]. Recent studies have indicated increasing complexity in the assortment of proteins that can bind the AP1 site. Both the Fos family (c-Fos, Fos-B, Fra-1, Fra-2) and the Jun family (c-Jun, Jun-B, Jun-D) have been expanded [19, 28]. All members of these families are capable of binding to AP1 sites, although a Fos member can bind only in association with a Jun family member. One common feature of all these factors is a leucine zipper domain, believed to be important in homo- or heterodimer formations.

Supershift assays were used to clearly establish that Jun-D, Jun-B and c-Fos proteins bind to the AP1 sequences contained within the human NQO_1 gene ARE [21, 29]. More recently, binding of Jun and Fos proteins to the human NQO_1 gene ARE was confirmed using *in vitro* translated proteins in gel mobility shift assays [26]. However, in a similar experiment, Jun and Fos proteins did not bind to the rat NQO_1 gene ARE [26]. The difference between human and rat NQO_1 AREs is in the presence and absence of a perfect AP1-binding consensus sequence that may be the determining factor for differences in proteins binding at the ARE. It is not known if the differences in regulatory protein binding to human and rat NQO_1 gene AREs is representative of species differences; this point remains to be investigated by analysis of human GST genes. Multiple factors including AP1 (c-Jun) and novel transcription factors have been shown to bind to the ARE enhancer element of the GST-P gene promoter [30]. Interestingly, these novel factors were shown to be present not only in differentiated HeLa cells but also in the non-differentiated F9 cells [30]. It is noteworthy that F9 cells do not express c-Jun and c-Fos but do express Jun-D and Jun-B [19]. The ARE-mediated CAT activity in F9 cells was thought to be because of novel transcription factors and not because of a Jun–Fos heterodimer [30]. Friling *et al.* [23] used gel mobility shift assays to demonstrate that *in vitro* translated c-Jun and c-Fos bind to the mouse GST-Ya gene ARE. In addition, they have shown activation of CAT gene expression upon co-transfection of ARE-CAT with c-Jun and c-Fos into mouse embryonic F9 cells. In contrast to the work of Friling *et al.* [23], Nguyen and Pickett [31] showed that *in vitro* translated c-Jun and c-Fos do not bind to the rat GST gene ARE and that TRE from human collagenase gene did not compete for binding of the nuclear proteins to the ARE. These observations led them to conclude that *trans*-acting factors that bind to the ARE are not Jun–Fos heterodimers. The same authors used photochemical cross-linking techniques to demonstrate that a heterodimer with subunit molecular masses of approximately 28,000 and 45,000 Da binds to the ARE [31]. The reason for the apparent discrepancies in the identity of regulatory proteins studied by different groups is not clear. Several factors, which include AREs from different genes, species differences, orientations of the two AP1 elements contained within the AREs, the presence and absence of ETS and other unknown binding sites within ARE, and differences in procedures of making nuclear extracts and gel shift assays, may have contributed to the discrepancies in the results. Further studies are needed to answer several interesting questions that include complete identification of regulatory proteins in the ARE–nuclear protein complexes and how these proteins bind at AP1 elements contained within the ARE. The methylation interference and protection assays have suggested that transcription factors bind in the major groove and involve contact with the CpG dinucleotide and the G residue within the 'TGAC' tetramer on the coding strand of the rat GST-Ya gene ARE [31].

Signal transduction mediated by ARE

The studies also raise important questions regarding the mechanism of signal transduction from bifunctional (β-NF) and monofunctional (BHA) inducers to the Jun/Fos and/or other unknown proteins that recognize ARE and increase the transcription of the phase II enzyme genes. The products of the proto-oncogenes Jun and Fos are expressed at low levels in most (but not all) cell types [19]. They are induced to higher levels transiently and rapidly by extracellular signals. They are believed to couple short-lived signals elicited by cell surface stimuli to long-term phenotypic changes by regulating expression of specific target genes containing AP1-binding sites. The external stimuli are known to control the activity of Jun and Fos proteins at the AP1-binding site by several mechanisms that include phosphorylation and cysteine modifications [32–36]. The involvement of protein kinase C in induction mediated by mouse GST-Ya subunit gene ARE was ruled out [23]. The involvement of tyrosine kinases in transcriptional activation of the NQO_1 and GST genes by bifunctional and monofunctional inducers is speculated because promoters of these genes contain AP1/AP1-like binding sites [37, 38]. However, this clearly needs to be determined by experimental analysis. At present, it is unrealistic to say that the mechanism of induction mediated by ARE will be the same as that observed in genes containing a perfect AP1 binding site (TRE) just because ARE contains AP1-like elements. Further experiments are needed to determine the similarities and differences in the mechanism(s) of induction mediated by the two elements. It will be interesting to know if β-NF, known to induce expression of reporter genes through ARE, could also increase expression by a single AP1 element with perfect consensus sequence (TRE) or for that matter whether inducers that function through the AP1 binding site (e.g. TPA) increase expression through ARE.

A second and more likely mechanism of induction could be similar, as has been demonstrated in bacteria. Two transcription factors, called oxyR and soxRS, have been shown to control the expression of multiple antioxidative enzymes in response to

H_2O_2 and reactive oxygen in bacteria [39, 40]. Bifunctional (β-NF) and monofunctional (BHA) inducers and endogenous compounds generate metabolites (redox signal) and reactive oxygen species [41–44], which by the process of reduction–oxidation could directly or indirectly modulate proteins for induced binding to the ARE or stimulate activity resulting in increased transcription of the gene. These investigations will help us understand the factors regulating oxidant and antioxidant responses in eukaryotic cells. Recently, Ref-1 protein has been identified as the major Jun–Fos redox activity in the HeLa cells and has been cloned [34–36]. Ref-1, a bifunctional protein, causes increased binding of Jun and Fos proteins to the AP1 site by reducing the cysteine in their DNA binding domain [36]. In addition, Ref-1 also possesses an apurinic/apyrimidinic (AP) endonuclease DNA repair activity [36]. Ref-1 also modulates several other transcription factors for increased binding to their respective *cis*-elements. There is no direct evidence for the involvement of Ref-1 in ARE-mediated induction. However, it is supported by the observation that several compounds that induce the CAT gene through ARE have the capacity to react with sulfhydryl groups (by oxidoreduction or alkylation), suggesting a mechanism involving protein thiol modifications [8]. Further investigations are needed to understand the role of Ref-1 or related protein(s) and/or protein kinases in signal transduction from β-NF to Jun and Fos proteins and/or novel transcription factors that regulate NQO_1 and GST gene expression. It is possible that induction of transcription of these genes in response to β-NF and BHA may involve the combination of two or more mechanisms that include modification of cysteines in DNA binding domains and phosphorylation by protein kinases of Jun and Fos and/or unknown proteins; this remains to be studied.

Recently, phenobarbital induction of GST-Ya and NQO_1 gene expression has also been shown to be mediated by the regulatory elements EpRE and ARE, respectively, and may involve Jun and Fos proteins [45].

Future perspectives

We think that expression of genes encoding one or more members of each class of detoxifying (phase II) enzymes and the proteins involved in drug resistance are coordinately regulated by ARE. The list of genes containing ARE sequences is likely to grow in the near future as more and more genes are sequenced and their promoters analysed. This is not surprising given the fact that a cascade of genes encoding proteins have to be activated to prevent the risk of oxidative stress, mutagenicity and carcinogenicity following exposure to drugs and carcinogens. In addition to the ARE, a second DNA element has been implicated in the induction of the phase II enzyme genes [1, 3]. This second element is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin) responsive and designated as the xenobiotic response element (XRE) [46]. The difference between ARE and XRE is in their capacity to induce phase I (cytochromes P450) gene expression. The XRE mediates induction of both phase I and II enzymes as compared with the ARE, which mediates induction of only phase II enzymes [16, 47]. The induction of phase I by XRE element may cause increased risk of mutagenicity and carcinogenicity, due to exposure to carcinogens, because carcinogens are activated by phase I enzymes into products that increase oxidative damage to DNA and membranes. Therefore, the ARE-mediated increase in phase II enzymes is the safest way to stimulate the chemoprotective power of the cells. Although the mechanism of induction through the XRE and aromatic hydrocarbon (Ah) receptor has been studied extensively, especially with regard to the cytochrome P450 genes [47, 48], the mechanism of signal transduction via the ARE has not been established. Pertinent questions concern differences in arrangement of AP1-like elements within the AREs, the role of ETS binding protein, species differences among AREs, complete identification of proteins binding at the ARE, the possible involvement of redox protein(s) and other redox messengers, and the manner by which these messengers receive signals from external stimuli (bifunctional and monofunctional inducers) and transmit this information in the form of increased transcriptional levels of NQO_1 and GST genes. It is reported [1] that dioxin-mediated induction of the NQO_1 gene expression does not involve ARE. However, several reports suggest otherwise [49, 50]. In these studies, dioxin is suggested to induce Fos and Jun, which may or may not be effective in the induction of the NQO_1 and GST genes by ARE, and remains to be investigated. In addition, research into the types of naturally occurring and synthetic compounds, which could act as potent inducers of the phase II enzyme genes through ARE, should contribute to the development of chemoprevention and chemotherapeutics.

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